# A colored view on quantitative pathology

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CHAPTER 6

# QUANTIFICATION OF EPITHELIAL PERCENTAGE IN OVARIAN TUMORS OF BORDERLINE MALIGNANCY

## COMPARISON OF A SEMIAUTOMATED AND AN INTERACTIVE QUANTIFICATION METHOD USING A TRUE COLOR IMAGE ANALYSIS SYSTEM

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**Abstract**

Quantification of stromal and/or epithelial compartments plays a role in the process of predicting the prognostic characteristics in a variety of epithelial tumors. The procedures for quantification vary from rough estimation to (semi)automated measurement. During the past years we have obtained experience in quantification procedures on various tumor types. The present study is meant as a methodological evaluation of two approaches to quantify the epithelial percentage (EPIT%) in a group of epithelial ovarian tumors of borderline malignancy, which were histologically classified on standard haematoxylin-eosin stained tissue sections. The material used in this study consisted of 29 tissue sections from 24 serous (SBT) and 22 from 18 mucinous (MBT) tumors. The epithelial compartment was stained with antikeratin antibodies on paraffin sections and haematoxylin was used as counterstain. In a subset the azan stain was used in an attempt to separate epithelial and stromal compartments in a different way. Using a true color image analysis system (IAS), two methods of EPIT% quantification were compared: one semiautomatic (IAS-EPIT%) and the other interactive (INT-EPIT%). The results showed that the IAS-EPIT%s were generally lower than those obtained interactively in the same image fields. This appeared to be a result of underestimation of the epithelial compartment and overestimation of the total tissue area in the semiautomatic procedure. The use of the azan trichrome stain showed an even stronger discrepancy and was, therefore, considered not applicable. The possible advantages of semiautomatic quantification, such as increased objectivity and reduced time consumption did not compensate for the loss of accuracy. We concluded that the application of interactive EPIT% quantification should be the method of choice in the type of tumor used here. The results confirm that there is no uniform rule for quantification procedures in biological material and that for each situation the method should be evaluated.

## Introduction

Morphology of ovarian epithelial tumors has an influence on patient morbidity and mortality figures. This is reflected in the World Health Organisation (WHO) classification. It is widely recognized that even amongst experienced gynaecological pathologists the inter- and intraobserver agreement in histological assessment of tumor type and grade is limited, approximating to 60-80% [2,5,143]. The volume percentage epithelium provides additional information for discrimination between borderline and malignant tumors of the ovary [59,143-146]. It is estimated through measurement of the area percentage of epithelium (EPIT%) in tissue sections [59]. This may be done (semi)automatically using digital image processing or interactively by point counting using a grid. There is a tendency to choose for (semi)automatic procedures because these are assumed to be faster and less tedious than the interactive method and therefore offer a practical advantage [59]. Mostly black-and-white systems are used. However, the use of real color as image feature can be useful [6,11,15,16]. At the present time true color image analysis systems are commercially available. Herewith, the selection of the area of interest can be done in images containing the spectral information the pathologist is used to work with.

Despite the advantage of automation it is stipulated that for routine laboratory use a well-developed digitizing interactive set-up can be as good or even better than an automated image analysis system [6,143]. To evaluate this a (semi)automatic quantification procedure was compared with an interactive method using a true color imaging system in a set of epithelial ovarian tumors of borderline malignancy.

## Materials and methods

*Patient material and staining*

Fifty-one formalin-fixed, paraffin-embedded tissue samples were selected from 42 cases of ovarian borderline tumors and used for quantification of EPIT%. From nine cases two tissue samples were selected, from the remaining 33 only one.

Using standard haematoxylin-eosin stained tissue sections 24 cases were classified as serous borderline tumors (SBTs) and 18 as mucinous borderline tumors (MBTs), according to the WHO criteria. From the 24 SBTs 29 tissue sections were used, whereas from the 18 MBTs 22.

Four μm thick tissue sections were, routinely, immunohistochemically stained for keratin (Keratin AE, 1:3, Boehringer, Germany) using a streptavidin-biotin peroxidase method (Dakopatts, Glostrup, Denmark). Diaminobenzidine was used as chromogen, resulting in a brown color for positive staining parts. Haematoxylin was used as counterstain.

Twenty-two tissue sections of 19 cases (16 tissue sections of 14 SBTs and 6 tissue sections of 5 MBTs) were also stained with a connective tissue stain, known as

Heidenhain's azan [137], which showed to provide good results in the quantification of EPIT% in breast carcinoma samples [43].

Because clinical evaluation was not the aim of this study the 51 keratin-stained sections as well as the 22 azan-stained sections were considered as separate cases.

*Image acquisition*

The VIDAS image analysis system (IAS) [63], capable of digitizing true color images was used for the semiautomatic as well as for the interactive method. The hardware consists of an Axioplan microscope with a halogen illuminator (Carl Zeiss, Oberkochen, Germany) fed by a stabilized power source, a single chip charge coupled device (CCD) color camera (WV-CD130, Panasonic, Matshushita Communication Co. Ltd., Yokohama, Japan) and a personal computer based on a 286 AT processor equipped with a frame grabber and expanded with a 287 mathematic coprocessor (Kontron Elektronik, Eching, Germany). The system uses VIDAS version 2.0, software capable of processing true color images, which are formed by a red, green and blue (RGB) image partition respectively. Images of 512×512 pixels were recorded using an objective with a magnification of 2.5× (numerical aperture=0.075). Measurements were started in the, visually identified, most epithelium rich area of the specimen. In this part of the tissue section a maximum of five images was used, corresponding to a measured surface of 23.7 $mm^2$. The maximum value over these images was taken to represent the volume percentage epithelium of the case [59].

*Semiautomatic quantification*

In the image processing method for the semiautomated assessment of the EPIT% the major focus is on segmenting the image into an epithelial area and a total tissue area. The quantification was performed without shading correction and by application of two sets of fixed threshold levels as previously described [42-44]. Two binary images resulted after segmentation: one representing the epithelial area, the other the total tissue area. Since artefacts may remain in both binary images [59] simple, additional processing steps were performed. Small artefacts in the background with an area of less than 20 pixels were removed in both binary images. Subsequently, small spaces between epithelial cells were closed when the area was less than 30 pixels whereas, small holes and tears in the binary "total tissue image" were closed when the area was less than 25 pixels. The ratio of the thus obtained number of pixels in the epithelium (E) and the number of pixels in the total tissue area (E+S), i.e. (E/E+S)%, gave the desired EPIT% (=IAS-EPIT%).

The semiautomatic quantification for the keratin- and azan-stained tissue sections

was similar except for the values in the sets of threshold levels.

*Interactive quantification*

For assessment of the interactively obtained EPIT% (=INT-EPIT%) a 256-point regular grid [128] was placed in an overlay over the same image fields as were used for the semiautomatic quantification procedure in a way previously described [42]. Without knowing the IAS-EPIT%, points overlying epithelium and stroma were counted. The ratio of the number of points overlying epithelium and the number of points overlying tissue resulted in the INT-EPIT% in the same way as described above. The procedures for the keratin- and azan-stained tissue sections were similar.

Because the visual identification of the most epithelium rich area, in which measurements were to be started, is restricted in objectivity and, hence, is subjected to effects of sampling we evaluated the reproducibility by repeating the interactive quantification procedure on ten tissue sections.

**Results**

Of the keratin-stained sections three could not be measured due to technical artefacts. It concerned three cases of SBT. The IAS-EPIT% and the INT-EPIT% of the remaining 48 keratin-stained tissue sections ranged from 8.5 to 70.3% (mean=31.9%; standard deviation=13.0%) and 7.0 to 76.0% (mean=38.3%; standard deviation=16.1%) respectively. Comparison of IAS-EPIT% and INT-EPIT% is displayed in Figure 6.1 and shows a modest, although significant, correlation coefficient ($r=0.75$, $p<0.001$). This figure shows that in general the semiautomatically obtained values are lower than those of the interactive method. Visual inspection of the segmented binary images showed that both epithelial and tissue segmentation not always gave satisfactory results. Underestimation of the epithelial part occurred (Figure 6.2), as well as overestimation of the total tissue area. As can be seen in Figure 6.1, it appears that the misestimation of IAS-EPIT% was more prominent in the MBTs (□) than in the SBTs (○). This difference is also reflected by the respective coefficients of correlation (MBTs: $r=0.67$, $p=0.0019$; SBTs: $r=0.78$, $p=0.0003$).

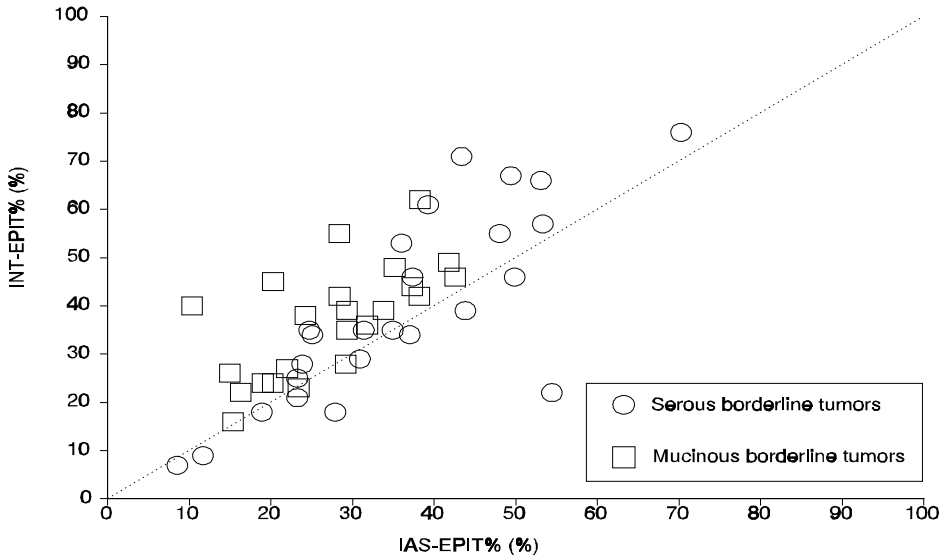


*Figure 6.1. Results of quantification of epithelial percentage (EPIT%) in 29 tissue sections of serous (○) and 22 of mucinous (□) borderline ovarian tumors. The IAS-EPIT%s and INT-EPIT%s were obtained in the same imagefields and represent the semiautomatically and interactively obtained values respectively.*

Figure 6.1 also shows one SBT in which the IAS-EPIT% is much higher than the INT-EPIT% (54.4% and 22.0%, respectively). This was a result of underestimation of the area of total tissue in the semiautomatic method (Figure 6.3).

Quantification of the azan-stained tissue sections was possible in only 7 of the 22 cases because in the others staining contrasts between the epithelial and stromal compartments were not sufficient to be segmented, neither semiautomatically, nor visually. Therefore, quantification on these sections was abandonded. The INT- and IAS-EPIT%s of the 7 cases where separation of the compartments was possible are displayed together with those of the corresponding values of the keratin sections in Table 6.I.

Repeat measurements of INT-EPIT% on ten keratin stained tissue sections to evaluate the possible influence of sampling errors in selection of the most epithelium rich area showed a high coefficient of correlation ($r=0.93$, $p<0.01$). However, despite high coefficients of correlation reproducibility may be restricted due to systematic differences [43]. Therefore, also analysis of variance (Friedman test) was applied. Herewith no significant systematic differences could be detected between the repeat measurements ($p=0.74$).

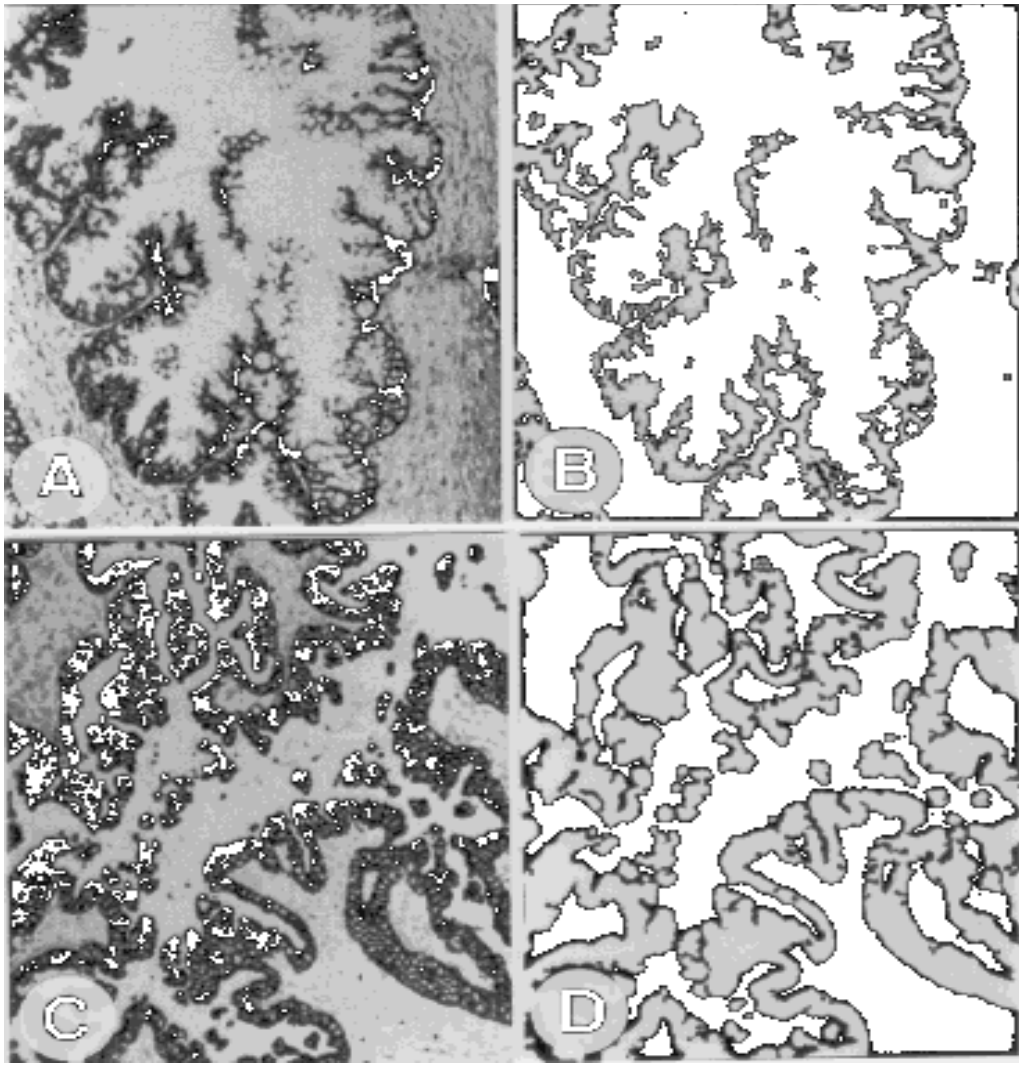


*Figure 6.2. Video printouts of parts of imagefields of a MBT (A) and a SBT (C) with corresponding binary images (B and D, respectively) representing the epithelial compartment. Panel A shows accumulation of intracellular mucus in the epithelial compartment resulting in lightly/unstained parts. This explains the underestimation of IAS-EPIT% found, which is visualized in panel B. Panel C shows that unstained areas occur in the epithelial compartment. This is a result of large nuclear structures. Although most gaps are closed during the additional image processing procedures described in the material and methods, some, located at the borders of the epithelial compartment remain open (D) resulting in underestimation.*

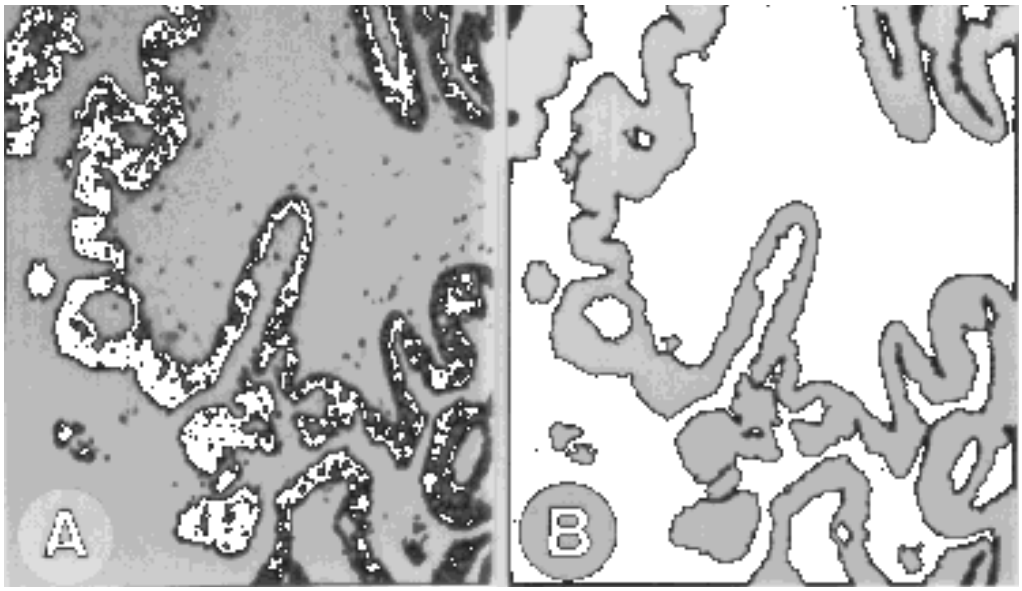


*Figure 6.3. Video printouts of a part of an imagefield of a SBT with a very "edematous" stromal compartment (A) and the corresponding binary image representing the total tissue area (B), which shows that automatic segmentation of stroma and background was not possible.*

**Discussion**

Quantification of EPIT% in ovarian tumors by using an automated method offers practical advantage over interactive morphometrical assessment [130]. For this, black-and-white (B&W) as well as true color IASs may be used. The true color IAS applied in this study, using a relatively low-cost single chip CCD camera, is suited for relatively simple quantification procedures, such as measurement of area percentage of staining [42,43]. This system was found to provide results comparable to those of interactive morphometry, which can be used as reference for evaluating image processing results [6,130], and to those of an IAS specifically dedicated to B&W image processing [45]. Moreover, it has the advantage that routinely stained tissue sections, resulting in more complex color images, may be used [43].

Both in diagnosis making and in quantitative assessments in common epithelial ovarian tumors following of protocol rules is of paramount importance, as it is in general in (quantitative) diagnostic pathology. Examples of issues concerned are: sampling (amount and localisation of samples), fixation time, tissue processing and staining, and selection of the area for measurement and the measurement method. In the type of quantification described in this study measurements may be affected

by effects of field selection [43,59,132]. Repeating the interactive measurement method on ten specimens showed that selection of the most epithelium-rich microscopic fields was performed in a reliable way. However, when comparing the values of the semiautomatic quantification procedure with those of the interactive method, which were obtained in the same image, the former were generally lower. Taking into account that the INT-EPIT%s can be used as reference [59], this implies that the results of the semiautomatic measurement deviate. Visual evaluation of the segmented images showed that this was mainly due to underestimation of the epithelial area and overestimation of the total tissue area, which were both non-systematic. Because IAS-EPIT% was derived from (E/E+S)% this explains the underestimation.

Table 6.I. Comparison of the INT- and IAS-EPIT%s of the azan-stained tissue sections where separation of epithelial and stromal compartments was possible, compared to the INT- and IAS-EPIT%s of the keratin sections

| Azan | | Keratin | |
|---|---|---|---|
| INT-EPIT% | IAS-EPIT% | INT-EPIT% | IAS-EPIT% |
| 14 | 23.80 | 18 | 18.91 |
| 30 | 23.28 | 29 | 30.92 |
| 34 | 21.75 | 35 | 31.37 |
| 52 | 24.71 | 46 | 37.36 |
| 48 | 57.86 | 53 | 36.02 |
| 52 | 37.79 | 55 | 48.03 |
| 74 | 58.02 | 71 | 43.37 |

INT-EPIT% = interactively obtained epithelial percentage
IAS-EPIT% = semiautomatically obtained epithelial percentage

Underestimation of the epithelial area occurred in the MBTs due to accumulation of intracellular mucus resulting in lightly or unstained areas in the epithelial compart-

ment (Figure 6.2 A and B). A comparable phenomenon, although to a lesser extent, was visible in some SBTs. Here unstained parts of the epithelial compartment were visible at the location of large nuclear structures (Figure 6.2 C and D).

Overestimation of the total tissue area was due to the presence of debris, like cellular and mucoid material, in the luminal areas of cystic spaces and glands. In many tumors the stromal compartment had an edematous aspect, which is a common finding [83], and therefore, was only lightly stained. Because the debris and these lightly stained stromal areas partly had comparable spectral values they could not be segmented, which, as a consequence, led to the overestimation.

The observation that the misestimation of IAS-EPIT% appeared to be more prominent for the MBTs than for the SBTs (Figure 6.1) may be explained by the presence of intracellular mucus, as well as the intraluminal debris, which is more prominent in MBTs [83].

The one case of SBT showing an IAS-EPIT% more than two times higher than the INT-EPIT% was a result of underestimation of the total tissue area. This was due to stroma with a highly edematous aspect. In the segmentation step this was considered as "unstained" background (Figure 6.3).
Experiments with different sets of threshold levels for segmentation of both epithelial and total tissue area to solve the problem of over- and/or underestimation did not provide satisfactory results.

Alternatives to overcome misestimation in the semiautomatic procedure may be found in other staining procedures, either histochemically or immunohistochemically [59]. The majority of the cases stained with azan, routinely used as connective tissue stain [137], did not provide the contrasting colors of the different compartments we were looking for. Although it provided good results to obtain EPIT% in breast carcinoma samples [43], it was not applicable in the present study. This demonstrates that what is suited for one tissue type does not necessarily apply to others.

Based on the results presented in this study, the choice will be in favor of a digitizing interactive set up, capable of providing true color images, when quantification of EPIT% in ovarian tumors is concerned. With this set-up EPIT% is relatively easy to assess in a reproducible way, as is also stated by others [6,143,147]. The skilled pathologist can perceive and analyse information-noise in the image and select what is to be in- or exluded from the measurement, an ability a digitizing automatic set-up lacks. Moreover, tissue sections stained with standard procedures can be used. These can be keratin stained sections, whenever routinely available for, for example, evaluation of early stromal invasion [148]. But mostly the omnipresent, standard haematoxylin-eosin stained tissue sections can and will be used.

Thus, this study underscores the stipulation, that for routine laboratory use a well-developed digitizing interactive set-up can be as good or even better than an

automated image analysis system.